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Mouse mammary tumor virus (MMTV) is used as an insertion mutagen in transgenic mice that express the *Wnt1* gene in their mammary gland, to produce additional events like activation of a second oncogene. Cloning cellular sequences flanking new proviral insertion from tumor 76 identified a common insertion locus for MMTV. Further analyses of this region identified *Fgf3* as the gene activated over a long distance by MMTV. *Fgf8* is another oncogene that I identified was activated in 10% of the mammary tumors from these infected Wnt1 transgenic mice. *Fgf8* encodes for at least seven different protein isoforms, three of which were isolated by us. These three isoforms (*Fgf8a*, *b* & *c*) differ in their NIH3T3 cell transforming abilities. *Fgf8b* induces programmed cell death of mammary epithelial cells. This apoptotic property is not confined to *Fgf8b* but is also seen when mammary epithelial cells are treated with purified human FGF proteins (FGF1, 2, 4, 6, & 9). Overexpression of BCL2 in the mammary epithelial cells delays the onset of FGF induced apoptosis. Female transgenic mice expressing the *Fgf8* gene in the mammary gland developed tumors with a latency of 5-6 months. These tumors were found to be adenocarcinomas: benign adenomas to invasive ductal carcinomas. Northern analyses of tumor RNAs show high expression of the transgene.

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FOREWORD

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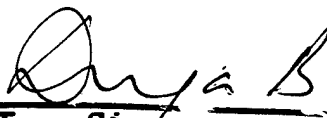

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INTRODUCTION

Breast cancer, like other cancers, results primarily from accumulation of genetic mutations. Many of the identified mutations associated with cancer result in the activation of proto-oncogenes or inactivation of tumor suppressor genes. In most cases, a single chromosomal aberration is insufficient to cause carcinogenesis but rather mutations in two or more genes are required. In order to understand the development and progression to cancer, it is imperative to identify not only the single mutations involved, but also synergistically acting groups of cancer related genes.

The role of retroviruses in viral-induced cancers has been well established in mice. For example, MMTV has proven to be a powerful tool for studying murine mammary tumorigenesis. MMTV is a B type retrovirus that is known to cause mammary adenocarcinomas in certain inbred strains of mice (e.g. C3H, BR6, GR) (1, 2). The tumor inducing property of MMTV is intrinsically related to an obligatory step in its life cycle, the insertion of a proviral copy of its genome into host cellular DNA. The integration is a mutagenic event for the host cells and, as a consequence, may lead to the transcriptional activation of closely linked proto-oncogenes by the mechanism of insertional mutagenesis (3). The activation of the proto-oncogene by MMTV contributes to the transformation of the cell and development of a tumor. A number of proto-oncogenes activated by MMTV in mammary tumors have been identified. They are *Wnt1*, *Wnt3*, *Wnt10b*, *Int2/Fgf3*, *Fgf3*, *Fgf-4* and *Fgf8* (4,5,6,7,8,9,11,17).

While the structure of these genes is known, less is known about their function. A common factor among the MMTV activated genes is that they all appear to play key roles in early embryonic development (12). To prove the oncogenic potential of *Wnt1*, transgenic mice containing the *Wnt1* gene under the control of an MMTV enhancer were generated. Both male and female transgenics developed mammary adenocarcinomas following a generalized mammary hyperplasia (13). The median latency of mammary tumor formation was ~ 5 months in female mice. Males developed tumors less frequently and later in life. The generalized hyperplasia, coupled with the long latency and the sporadic nature of the tumor formation, suggest that *Wnt1* contributes to but is not sufficient for mammary tumorigenesis in these mice. Activation of *Wnt1* is probably an early event in the process of tumor formation; therefore other events, presumably genetic, are necessary for tumor progression.

In an attempt to identify genes acting in synergy in the multistep process of murine mammary tumorigenesis, these *Wnt1* transgenic mice were mutagenized by infection with MMTV (14,15). The strategy was that since MMTV transcriptionally activates proto-oncogenes by insertion of its own DNA near them (2), one could possibly identify additional oncogenes that oncogenically cooperate with *Wnt1* by tagging them with viral DNA. Activation of the cooperating oncogene would therefore confer a growth advantage and would presumably produce a tumor composed mainly of cells that are clonally derived from the cell bearing the proviral insertion. Implicit in this hypothesis was the expectation of a reduction in tumor latency. As predicted, in MMTV infected *Wnt1* transgenics the median latency of tumor formation decreased from ~5 months to 2.5 months and the number of tumors per mouse increased (15). Southern blot data reveal that most of these tumors contained clonal tumor-specific proviruses in addition to the endogenous proviruses found in laboratory mice (15). The advantage of this approach over other mutagenesis procedures is that tumors arising due to proviral insertions contain proviruses physically linked to the activated proto-oncogenes, forming a molecular tag which permits easy identification and cloning of the activated genes (2).

Analysis of the tumor DNAs derived from infected *Wnt1* transgenic mice by Southern blotting showed that at least 80 of 128 tumors (59%) contained clonal MMTV-specific proviruses (15). These tumors were examined for the insertional activation of proto-oncogenes known to be activated by MMTV: *int2/Fgf3*, *hst/Fgf-4*, *int-3* and *Wnt-3* (2,5,7,8). Approximately 45% of these tumors contained insertionally activated *int2/Fgf3* and/or *hst* (15). These results show the cooperation of *int2/Fgf3* and *hst* with *Wnt-1*, which strongly corroborates prior findings indicating the same cooperation (16). I (in collaboration with a post-doctoral fellow Craig MacArthur) recently identified another member of the FGF family of growth factors that is insertionally activated by MMTV in 8 of 80 mammary tumors with clonal tumor-specific proviral insertions (17). This gene (*Fgf-8*) was cloned from one of the tumors that had a single tumor specific proviral insertion as described in the methods section. *Fgf8* is transcriptionally activated in the tumors from a silent state (17). This is the third member of the FGF family to be activated in this system, indicating that *Fgfs* and *Wnts* are strong collaborators in inducing mammary tumors.

As we have already demonstrated, this infected *Wnt1* transgenic system can be used to identify novel or/and unexpected oncogenes that are involved in mammary tumorigenesis, thereby demonstrating oncogenic cooperation with *Wnt1* and elucidating the multiple steps involved in murine mammary tumorigenesis. At the time of the original proposal, we still had ~ 55% of the mammary tumors from infected *Wnt1* transgenic mice with new proviral insertions in which the known targets of MMTV mutations are not affected.

My specific aims:

1. Isolation and identification of proto-oncogenes (novel and unexpected) insertionally activated by MMTV in tumors of infected *Wnt1* transgenic mice.
 - Identification of proviral-cellular junction fragments.
 - Clone cellular sequences flanking the proviral insertion
 - Locate and isolate the activated gene in the locus using Northern blot and exon trap strategies.
 - Determine the expression pattern of the gene in normal tissues and in tumors.
2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.
 - Demonstrate the oncogenic potential of the isolated proto-oncogene in cell culture transfection assays.
 - Demonstrate the gene's oncogenic potential *in vivo* using transgenic mice.
3. Demonstration of the cooperativity of *Wnt1* with the proto-oncogene that is activated by MMTV.
 - Demonstrate cooperativity by cotransfection of C57MG cells.
 - Obtain definite proof of cooperativity by generating bitransgenic mice.

BODY OF ANNUAL REPORT

Specific Aim 1. Isolation and identification of proto-oncogenes insertionally activated by MMTV in tumors of infected *Wnt1* transgenic mice.

Task-1: Cloning the junction fragment (s), and isolation of the gene (s) activated by MMTV insertions.

Summary of previous results (report-1995, 1996)

In my previous progress report, I described the identification and cloning of a proviral-cellular junction fragment from tumor #76 from infected *Wnt1* transgenic mice. Screening of the tumor panel by Southern analysis using cellular probes derived from this cloned region detected insertions in 12 of 85 tumors within this locus, indicating that this is a new common insertion locus for MMTV. Northern analysis and exon trapping procedures failed to identify any coding sequences within the cloned region (Figure 1). The locus was mapped to the distal region of mouse chromosome using the Jackson laboratory Backcross DNA Panel Map service (23). Southern and northern blot analyses of these tumors identified *Fgf3/int2* as the gene activated by MMTV in the tumors with insertions in this locus.

Since the oncogenic potential of *int2/Fgf3* has already been demonstrated, we and others have shown *int2/Fgf3* to be a strong oncogenic collaborator of *Wnt1* in promoting mammary carcinogenesis (11,15,16). As a consequence of this fact, I slightly altered the focus of my proposal from that originally described. This change does not affect or change my primary goals towards identifying genes involved in the multistep process of mammary tumorigenesis, nor does it alter the specific aims and focus of research described in the original proposal. The only difference was that further characterization of the identified gene *int2/Fgf3* was not performed, instead I pursued the characterization of a gene (*Fgf-8*) that I had previously identified to be frequently activated by MMTV proviral insertions in these infected *Wnt1* transgenic mice (17).

Specific Aim 2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.

Task-2a: Demonstration of the oncogenic potential of the isolated proto-oncogene in cell culture assays.

Summary of previous results (report 1996)

Fgf8 consists of at least six exons and codes for at least seven protein isoforms, due to alternative splicing of the primary transcript (17,18). We analyzed the oncogenic potentials and differences in the biological activities of three isoforms (8a, 8b, and 8c) in NIH3T3 cells and showed that the isoform Fgf8b was highly transforming in NIH3T3 cells and highly tumorigenic in nude mice, while the other two isoforms showed moderate to low transforming potentials (22). Additionally, I also tested the human isoforms of FGF8 for their oncogenic capabilities (cDNAs obtained from Dr. P. Roy-Burman). FGF8b was found to be highly transforming when compared to FGF8a or FGF8e, which show low to moderate degrees of transformation in NIH 3T3 assays and anchorage independent growth assays.

Biological effects of murine Fgf8 isoforms on mammary epithelial cells

In my previous report I had described an interesting property that I had discovered while analysing the differences in the biological activities of the Fgf8b protein isoforms. I had found that mammary epithelial cells either over expressing Fgf8b or when treated with 8b protein containing conditioned medium, underwent apoptosis. I demonstrated that the cell death was indeed due to apoptosis by Hoescht staining and DNA ladder analyses. Since this was the first observation so far that Fgfs could cause apoptosis of mammary epithelial cells, to better test our results, we partially purified Fgf8b using a heparin sepharose affinity column and demonstrated induction of apoptosis by the partially purified Fgf8b protein.

In the last year I have done more experiments on this property of Fgf8b. I will describe the results in this report. Eventhough these experiments were not originally proposed, they are highly significant to my rposal, and also to the understanding of oncogenic cooperation in this system.

Induction of apoptosis by several members of the FGF family of growth factors.

To test if the phenomenon of apoptosis was specific to only Fgf-8b or was also seen with any other FGF family member(s), I used purified human FGF1,2,4,5,6,7, and 9 (R&Dsystems) to treat C57MG cells. 100ng/ml of the FGF proteins was added to the cells at 40%confluence with 0.1mg/ml of Heparin. NIH 3T3 cells were used as a control to assay the activity of the protein. Cells treated with FGF1, FGF2, FGF4, FGF6 and FGF9 showed morphological transformation and apoptotic cell death similar to Fgf8b. FGF2, FGF4 and FGF9 showed this effect even in the absence of heparin. FGF5 did not show any changes at concentrations of 200 ng/ml. Cells treated with 500 ng/ml of FGF5 showed moderate transformation but no apoptosis, and cells treated with 1mg/ml of the growth factor showed both transformation and apoptosis. The only growth factor that was tested but did not show any activity was FGF7. Even 1mg/ml of FGF7 did not show any changes in morphology (Table 1).

Neutralization experiments using a polyclonal antibody against human FGF2 completely blocked the apoptosis seen on treatment with FGF2 in C57MG cells (Figure1). Cells treated with FGF2 showed transformation and apoptosis by days 3-4. However, the cells treated with FGF2 that was pre incubated with the antibody did not show any alteration in cellular and nuclear morphology, indicating that the apoptosis seen was indeed a result of treatment with FGF2/FGFs.

To address the possibility that this effect is not something specific to this cell line, we tested several other mammary cell lines (normal and tumor) for induction of apoptosis by FGF2, FGF4, FGF6 and FGF8. Three cell lines (C127I, BMG, GR) resembled C57MG in all respects; cellular transformation followed by apoptosis. HC11 cells showed some degree of apoptosis but cell death was not complete as seen in the other cell lines. The human breast carcinoma cell line MCF-7 when treated with the different FGFs did not undergo programmed cell death (Table 2). One explanation to this unresponsiveness to FGF signals is that MCF-7 cells are known to express high levels of the anti-apoptotic gene product Bcl-2. Therefore, Bcl-2 may be inhibiting or blocking the apoptotic signal provided by the FGF. Alternatively, MCF-7 being a carcinoma cell line could have gone through several genetic alterations, some of them may result in providing survival functions for the cells.

Inhibition of FGF induced programmed cell death by Bcl2

The Bcl2 protein is known to block apoptotic cell death induced by various agents (31). However, Bcl-2- independent apoptotic pathways are also known. We tested to see if Bcl-2 could inhibit FGF induced apoptosis of mammary epithelial cells. C57MG cells were transfected with an expression vector containing the *Bcl-2* cDNA. Stable clones expressing the Bcl-2 gene product were generated and treated with FGF2, FGF4 and FGF6. Stable clones expressing the

vector alone was used as controls. The treated cells were observed for a period of 4 days. The control cells (clones transfected with vector alone) showed morphological changes within 12-16 hrs after treatment. By 24 hr's many cells started dying and coming off the plate. By day 3 after treatment approximately 80% of the cells were apoptotic. In contrast, the Bcl-2 clones remained viable and were morphologically transformed from a flat cuboidal morphology to a more elongated spindle shaped appearance (Figure 2). No apoptosis was observed suggesting that Bcl-2 can block the apoptotic signal from FGF. However, on days 4 and 5, when the control cells were completely dead, the Bcl-2 clones started showing signs of apoptosis. This result could be explained by the possibility of transient expression of the protein from the vector or poor stability of the protein. It could also be possible that Bcl-2 does not completely inhibit apoptosis but delays the onset. This effect has been previously described for Bcl2 (31).

Task-2b: Confirmation of the oncogenic potential *in vivo* using transgenic mice.

The *Fgf8* transgene:

The transgene for the *Fgf8* transgenic mice was directly cloned from a tumor (tumor 86) that has an MMTV insertion very close to the 5' end of the *Fgf-8*. This particular insertion is in a "promoter insertion" orientation, i.e., it is upstream from the initiation codon in the same transcriptional orientation. This tumor expresses high levels of *Fgf8*RNA and hence, the transgene is likely to express well. The transgene contains the 3' MMTV LTR, and the entire genomic *Fgf8* gene in a single DNA fragment of ~13 Kb. The DNA was size selected on an agarose gel and cloned into a lambda vector (Lambda Dash) and the resulting library was screened using both MMTV specific and *Fgf8*specific DNA fragments as probes. The 13kb fragment was excised from this vector and used for microinjection of fertilized mouse eggs. The injections were performed at the transgenic core facility of the University of Southern California.

Fgf8 transgenic mice:

The founder animals generated were screened by Southern blots of the tail DNAs for the presence of the transgene. We had three transgenic founders that contain the MMTV/*Fgf8* transgene. These animals were bred to normal BALB/c mice to generate independent lines. There were two females and one male founder. We were able to get several offsprings from the transgenic male and Balb/c female. However, we had problems with the transgenic females. These animals were very bad mothers. They had really small litters and were unable to nurse their young. They ate their pups right after they were born at the time of cleaning. We tried to foster feed the pups, but were unable to get any offsprings. Meanwhile the female founders started developing tumors. Since these tumors were large and seemed to have fluid filled cysts, we removed the tumors and tried to breed them again. Unfortunately I was unable to establish a line from these animals. However, since both the female mice developed tumors, I analyzed the tumors for expression of *Fgf8*, and looked at the histology of the mammary gland and tumors. The offsprings from TG#18 (male founder) did not express the transgene. I looked at several animals but could not detect any expression by northern analyses of mammary gland, salivary gland, testes RNAs.

Tumor Development:

Mammary tumors started developing in transgenic 17 after 5 months. Initially a large tumor was present in the right (R1) gland, with smaller lumps in several other glands. Soon all the mammary glands were involved and developed into tumors. We sacrificed the animal at 8 months and removed all the tumors, and normal tissues. At this time we also saw that the ovaries were enlarged,. all the tissues were frozen for RNA analysis and part of it formalin fixed for

histology. The female founder TG#3, was similar to TG#17, however it had undergone only one pregnancy. This animal started developing tumors at about 7 months. In this animal too, all the glands were involved. Even though at the time of death, the tumors were of various sizes ranging from large cysts to solid tumors and small lumps.

Expression of the transgene in the mammary gland and tumors.

Total RNA was extracted from the frozen tumors, surrounding mammary gland, salivary gland and ovaries using the guanidium isothiocyanate/acid-phenol method (30). , RNAs were resuspended in 50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 0.25 mg/mL ethidium bromide, 0.66 M formaldehyde and 50% (v/v) formamide, denatured at 65°C for 5 minutes, and electrophoresed in 0.8% agarose gels with the same running buffer minus ethidium bromide and formamide at 30-45 V for 6-14 hours (38). Following photography, the gel was capillary blotted overnight to nylon. The blots were UV-crosslinked (11), prehybridized for 1-2 hours at 65°C, and hybridized at 65°C with ³²P-labeled Fgf8b cDNA probe. The hybridization buffer was 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% bovine serum albumin (Fraction V), 7% sodium dodecyl sulfate (SDS), and 30% (v/v) formamide. Blots were washed in 40 mM sodium phosphate (pH 7.2), 1% SDS, and 1 mM EDTA at 65°C. Blots were exposed to Kodak XAR-5 film with intensifying screens at -80°C.

The signals on the membrane were so strong that the blots were developed within 3 hrs. Very high expression of Fgf8b RNA was seen in the tumors. Expression was also detected in the mammary gland RNA and interestingly in the ovaries. Even though normal expression of Fgf8 can be found in adult ovaries. It could be detected only with a northern blot containing 20 µg of Poly(A) RNA (17), whereas I was able to see a signal with 10 µg of total RNA with 3 hrs exposure (Figure 3).

Histology of the tumors

The tumors were formalin fixed, paraffin embedded and sections were stained with Hematoxylin and Eosin. The stained sections were analyzed microscopically. The tumors from the transgenic animals were ductal adenocarcinomas. They showed varying degrees of pathogenicity, ranging from benign adenomas to clearly invasive papillomas (Figure 4). The mammary gland tissue were hyperplastic. The sections were analyzed by Dr. Shimada from the pathology lab of Childrens Hospital Los Angeles. Interestingly, the section of the ovaries showed stromal hyperplasia (Figure 5)

Specific Aim 3: Demonstration of the cooperativity of *Wnt1* with the proto-oncogene that is activated by MMTV.

Task 3a:• Demonstrate cooperativity by cotransfection of C57MG cells.

Since Fgf8b by itself is highly transforming in NIH 3T3 cells, and induces apoptosis of C57MG mammary epithelial cells, It would not be possible to demonstrate oncogenic cooperativity as defined in the classical experiments. However, we could speculate that the cooperation may not be the synergistic action of two oncogenes, but maybe Wnt 1 provides survival signals to the cells, that have received the death signal from Fgf8b and hence cooperation towards cellular proliferation leading to transformation. I am currently testing this hypothesis to see if in the presence of Wnt1 the cells overexpressing Fgf8b or when treated with FGF proteins survive and continue to proliferate.

Task 3b:• Obtain definite proof of cooperativity by generating bitransgenic mice.

Unfortunately we have had a severe set back in our experiments, because of our inability to generate Fgf8 transgenic lines. We are currently in the process of injecting more embryos with the same construct. We are also in the process of making other cDNA constructs for microinjection. Once we have these, we will try to obtain transgenic lines. This time we are planning to use several approaches to foster the pups as soon as they are born. We have some very good information about the same from Jackson labs and are hoping that we will not face severe problems again. Once we have these animals we will mate them with the Wnt10b animals that we already have in the lab (Shackleford et.al. unpublished results).

CONCLUSIONS

A proviral-cellular junction fragment was identified and cloned from a tumor from infected Wnt1 transgenic mice. Twelve of eighty five tumors tested had MMTV insertions within this locus. Analyses of these tumors showed that this newly identified cluster of MMTV insertions activate the previously characterized proto-oncogene *Fgf3*, over a long range (~20kb upstream of the gene). Since this gene has already been identified as an oncogenic collaborator of Wnt-1, I changed my focus to characterization of another gene *Fgf8* that I had previously cloned from a tumor from infected *Wnt1* transgenic mice, and identified to be activated from a silent state in 10 % of the tumors. This gene encodes at least seven different protein isoforms, three (Fgf8a,b and c) of which were isolated in our lab.

Characterization of the biological activities of the different isoforms in mammary epithelial cells identified a new property (apoptosis) for the isoform Fgf8b. Stimulation of C57MG cells by Fgf8b containing conditioned medium resulted in apoptotic cell death as shown by characteristic nuclear changes and DNA laddering. This finding was further proved by demonstration of apoptosis using partially purified Fgf8b protein (heparin sepharose affinity chromatography). This property was not confined to Fgf8b alone, but several other members of the FGF family of growth factors show the same effect. Apart from C57MG cells, other mammary epithelial cells also undergo apoptosis when induced by FGFs. Overexpression of Bcl2 in C57MG cells delayed the onset of apoptosis induced by FGFs.

To demonstrate the oncogenicity of Fgf8 in vivo, the mouse *Fgf8* transgene was cloned from a tumor with insertion in this locus (tumor 86). Three founders were obtained and unfortunately we were unable to establish any transgenic lines. We are currently injecting mouse embryos to generate more transgenic mice. The female founders developed tumors in 5-7 months. The tumors and surrounding mammary tissue highly expressed the transgene. The tumors were found to be ductal adenocarcinomas and stromal hyperplasia of the ovaries was also observed.

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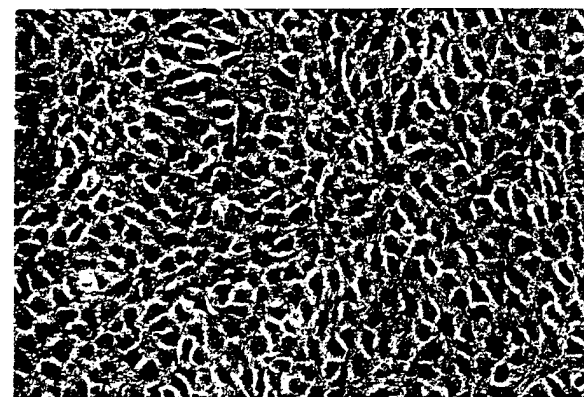
Apoptosis of mammary epithelial cells induced by several members of the FGF family .

FGF	Transformation of NIH3T3 cells	Apoptosis of mammary epithelial cells
FGF-1	+++	+++
FGF-2	+++	+++
FGF-4	+++	+++
FGF-5	+++	+/-
FGF-6	+++	+++
FGF-7	+++	-
FGF-8	+++	+++
FGF-9	+++	+++

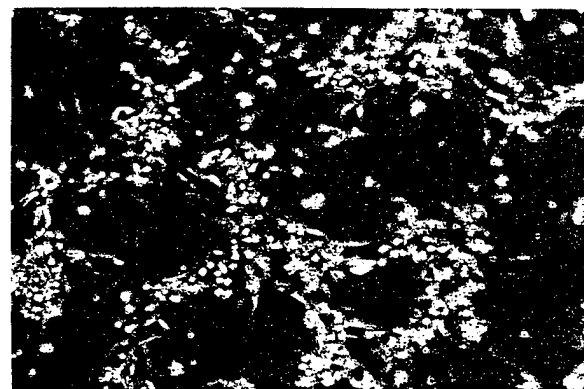
TABLE 1

Neutralization of the induction of apoptosis by FGF2 using a specific anti-FGF2 antibody

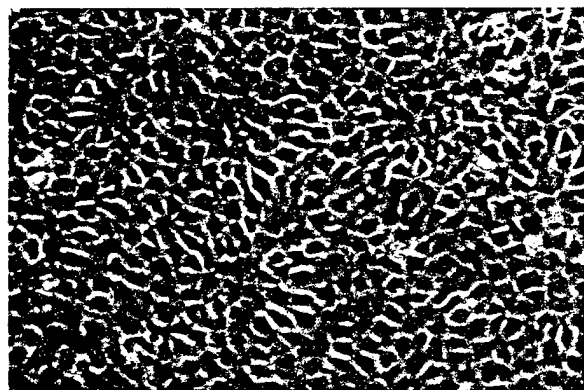
FIGURE 1



anti-FGF2 ANTIBODY
+ FGF2 100ng/ml



FGF2 100ng/ml



anti-FGF2
ANTIBODY

C57MG

Mammary epithelial Cells Apoptosis

C127I	+++
HC11	+/-
BMG	+++
GR	+++
S115	-
MCF-7	-

Fibroblasts

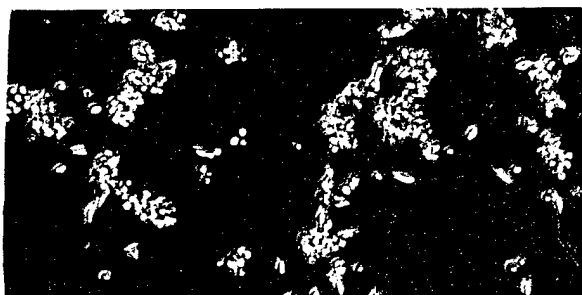
NIH3T3	-
Rat-1	-

TABLE 2

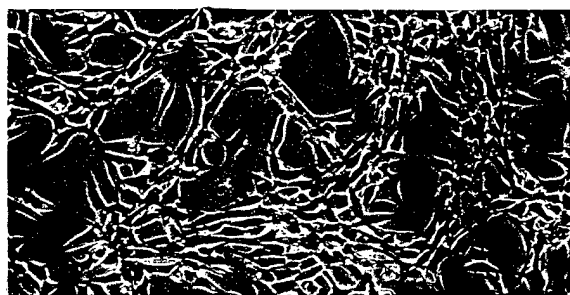
FIGURE 2

Rescue of apoptosis by human BCL2

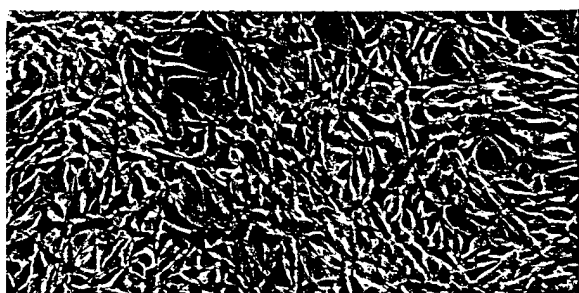
VECTOR



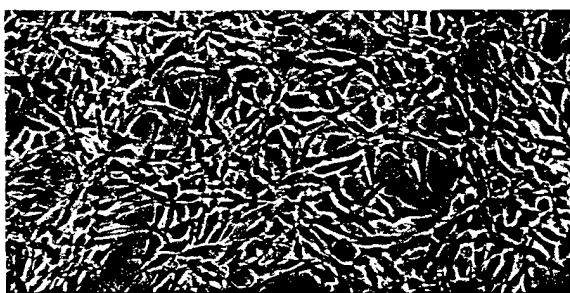
BCL2 clone 1



BCL2 clone2



BCL2 clone3

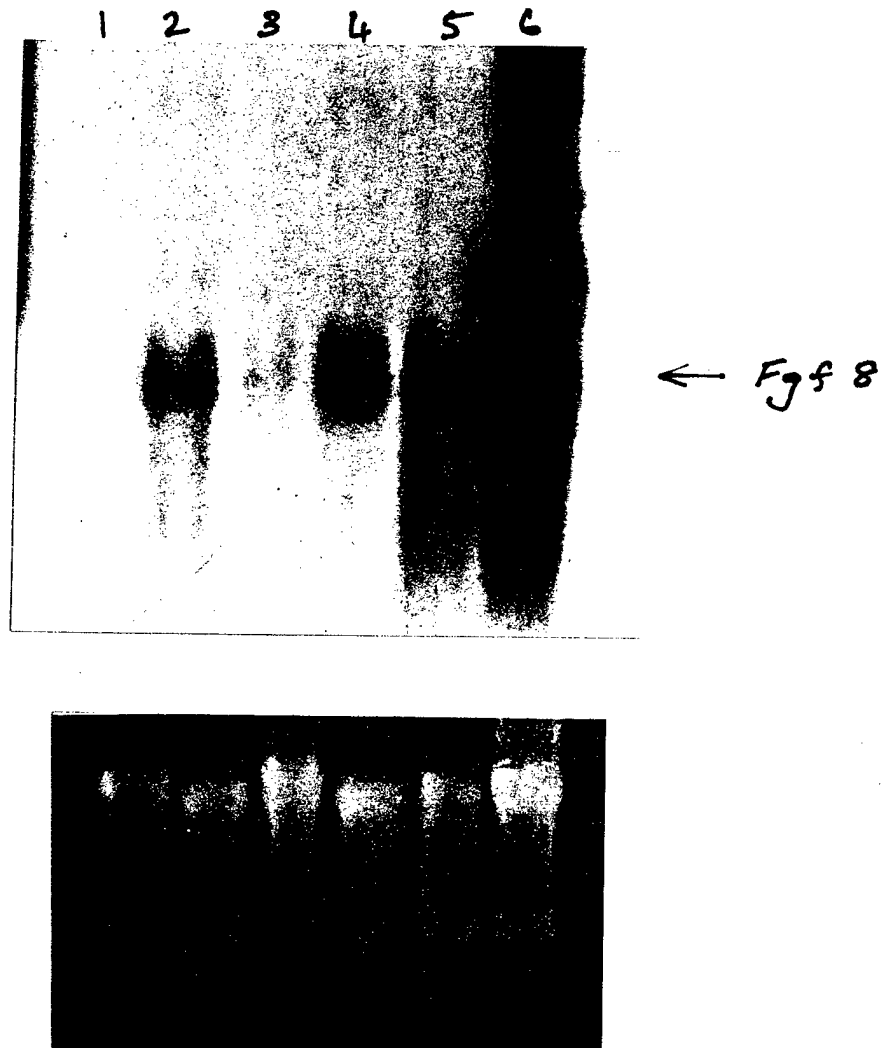


* Treated with human FGF2 protein 100ng/ml

- Overexpression of human BCL2 protein in C57MG mammary epithelial cells transiently rescues the cells from apoptosis induced by FGFs.

FIGURE 3

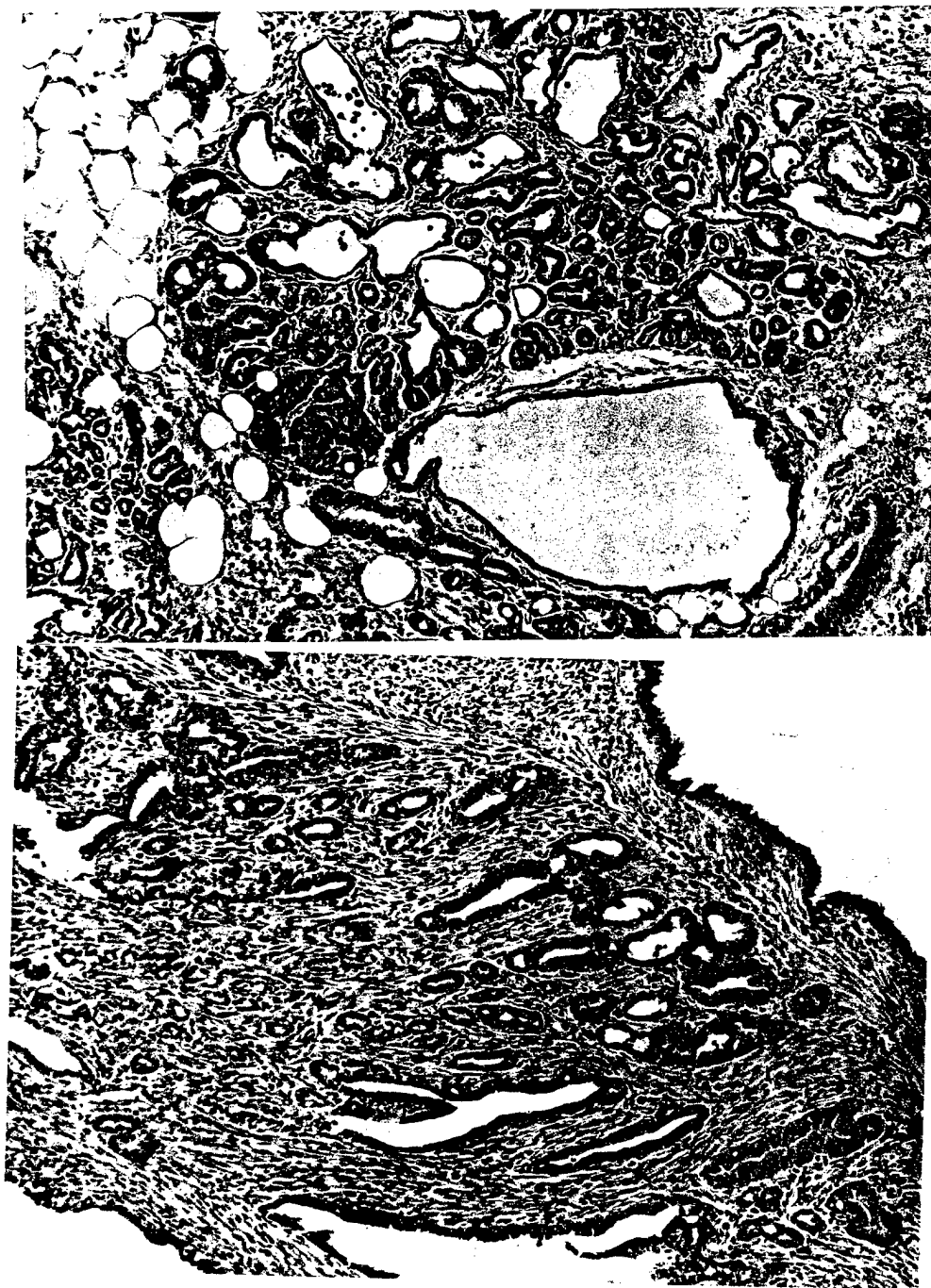
Northern Blot of RNAs from *Fgf8* transgenic mice



Northern blot of RNAs from tumors and some tissues. Lane 1- salivary gland
Lane 2- mammary gland, Lane 3- ovaries, Lane 4 -6, mammary tumors.

Figure 4

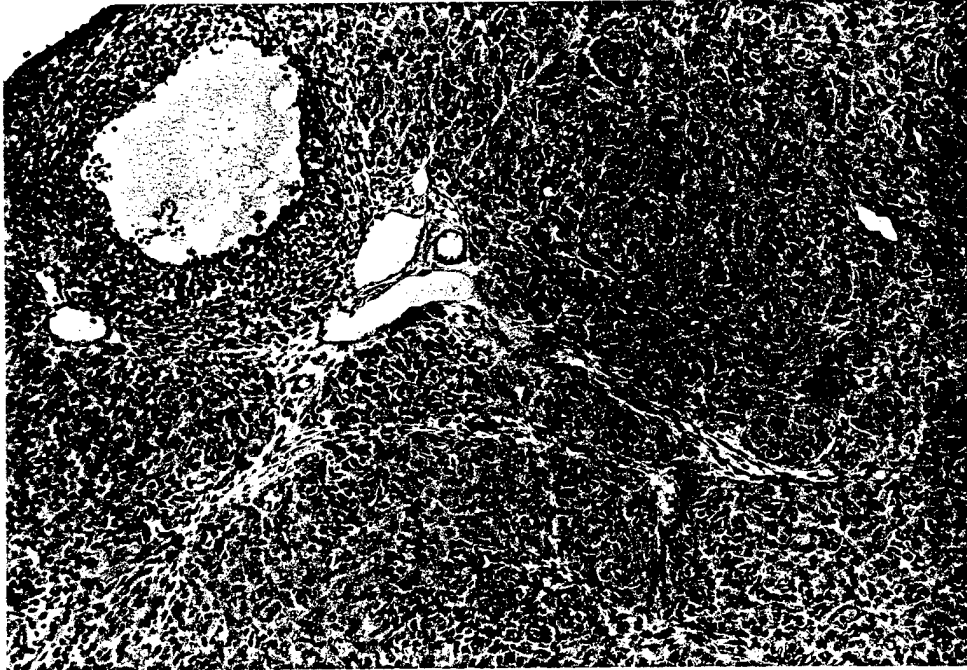
Histology of tumors from Fgf8 transgenic mice



Sections of tumors from Fgf8 transgenic mice. H&E staining of the sections, 100x magnification
A. benign adenoma, showing ductal proliferation
B. Invasive ductal carcinoma

Figure 5

Section of the ovary from Fgf8 transgenic mouse



H&E staining of the section showing stromal cell hyperplasia. (100x magnification)